ANAEROBIC GLYCOLYSIS IN AMPHIBIAN DEVELOPMENT[1]

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Under anaerobic conditions, the embryos of frogs probably degrade carbohydrate to lactic acid by a sequence of chemical reactions of the Embden-Meyerhof type. The operation of the sequence (glycolysis) presumably is able to generate some or all of the energy required for cleavage under anaerobiosis (Brachet, 1934). It may even meet the energetic demands of anaerobic gastrulation (Gregg and Kahlbrock, 1957), although Brachet (1960) has pointed out that the evidence is conflicting. In any case, it would appear to be an important device for sustaining embryos in straitened respiratory circumstances, of frequent occurrence in the interior of large clumps of naturally oviposited eggs. It is surprising, therefore, to find that little attention has been given to systematic study of the glycolytic capacities of frog embryos. A few papers on the subject exist in the literature. But some of these (Lennerstrand, 1933; Brachet, 1934) were published before the exigencies of rearing embryos under strictly *aerobic* conditions were understood or overcome, and are subject to still other criticisms noted by Cohen (1954). Others, including Cohen's paper and the earlier one of Barth (1946), cover only rather narrowly circumscribed morphogenetic periods. We are thus without a complete picture of the glycolytic behavior of pre-hatching frog embryos.

This paper has two purposes. The first is to take some steps toward filling the hiatus mentioned above, by surveying the glycolytic activity of developing *Rana pipiens* embryos. The second is to explore more thoroughly than before the reduced glycolytic activity exhibited by gastrula-arrested hybrid embryos obtained by fertilizing *Rana pipiens* eggs with *Rana sylvatica* sperm. Hybrids of this type were first studied by Moore (1946), from a morphological point of view. Later analysis has provided a rough outline of their physiological or biochemical peculiarities: its current status may be ascertained by consulting Barth and Barth (1954), Gregg (1957), Gregg and Kahlbrock (1957), Gregg and Ray (1957) and Gregg (1960).

Methods

Embryological

Developing embryos were obtained by stripping eggs from pituitary-activated *R. pipiens* females into suspensions of active *R. pipiens* or *R. sylvatica* sperm. After not more than one hour, the developing eggs were separated into small groups of 20–40 members each, and distributed into several large fingerbowls. Each fingerbowl contained 100–200 ml. of 10% amphibian Ringer's solution,

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without phosphate or bicarbonate. Development was allowed to proceed at 10° C., the medium being renewed every two days or so. Immediately before using them as experimental subjects, the embryos were freed of their jelly-coats with the aid of jeweler's forceps.

Chemical

Anaerobiosis was obtained with the help of the apparatus depicted in Figure 1. Twenty jelly-free embryos, along with enough rearing-medium to make a total volume of 2 ml., were placed in each of several 25-ml. Erlenmeyer flasks. The ground glass neck of each flask was closed with a No. 1 two-hole rubber stopper bearing glass inlet and outlet tubes. The flasks were set in the clips of a Dubnof

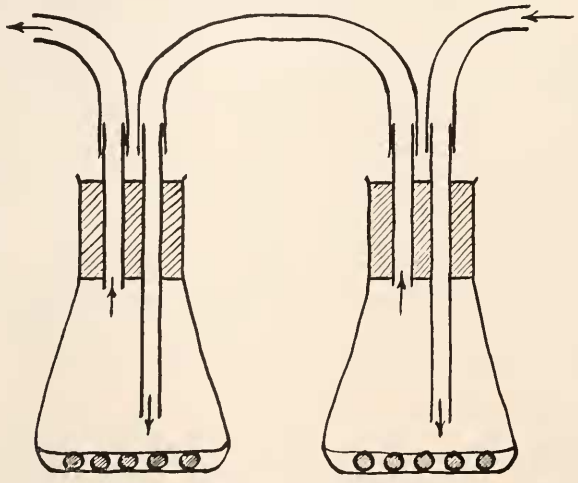

FIGURE 1. Apparatus used to obtain anaerobic embryos. See description in section on chemical methods. Arrows indicate direction of gas flow.

shaking bath running at 24° C., and connected in series with short lengths of rubber tubing. The shaking mechanism was then set in motion at a rate of 85 cycles per minute. At time zero, a source of washed 95% N_2: 5% CO_2 was connected to the inlet tube of the first flask, and gassing was allowed to proceed at a rate of one liter per minute for the duration of the experiment.

At the ends of chosen intervals, flasks were removed from the distal end of the train and their contents were emptied into 12-ml. graduated centrifuge tubes, each containing 0.5 ml. of 30% trichloracetic acid. The volumes were adjusted with 6% trichloracetic acid to values depending on the expected amounts of lactic acid, and the tubes were placed in a Deepfreeze. After freezing and thawing, the embryos were homogenized with a ball-tipped glass rod, and protein-free extracts were obtained by centrifuging.

The amounts of lactic acid in the protein-free extracts were estimated by a modification of the method of Barker and Summerson (1941). One-ml. aliquots of protein-free extract were treated with equal volumes of 2.5% $CuSO_4 \cdot 5H_2O$ and

125-mg. portions of $Ca(OH)_2$ to remove interfering substances. After centrifuging, 0.5-ml. aliquots of supernatant were combined with 3-ml. aliquots of concentrated sulfuric acid-copper reagent and heated to convert the lactic acid to acetaldehyde. Polyindophenol reagent was added, and the resulting color intensities were read at 560 mμ with the help of a Beckman Spectronic colorimeter. Standards were prepared with lithium lactate.

Terminological

Developmental stages were determined by reference to the data of Shumway (1940), which standardize the course of *R. pipiens* development at 18° C. Regardless of their actual temperature histories, embryos in a given Shumway stage have been assigned the corresponding standard age in hours. Hybrid embryos, even after the curtailment of morphogenesis at Stage 10, have been assigned the same stages as *R. pipiens* control embryos of the same female parentage.

Results

(1) Aerobic embryos contain negligible amounts of lactic acid.

This finding was established directly by a few analyses of *R. pipiens* embryos reared under the conditions described in the section on embryological methods. The results are summarized below:

Clutch	Stage	Standard age	Lactic acid, μg. per embryo
B	3	3.5	0
A	9	21	0.06
A	12	42	0
C	19	118	0.15
C	20	140	0.20

A similar result is implicit in the data presented in Figure 2 and Figure 3, where the values for lactic acid production under anaerobiosis extrapolate satisfactorily to a value of zero at time zero.

These results are in contrast to those of Lennerstrand (1933) and Brachet (1934), who reported much higher aerobic lactic acid values. Barth (1946), however, was able to show that the partial anaerobiosis induced by crowding will result in the production of large amounts of lactic acid, and both he and Cohen (1954) showed that when care is taken to keep embryos well-aerated, there is little or no lactic acid produced. It may be, therefore, that the embryos of Lennerstrand and Brachet were not reared under strictly aerobic conditions.

(2) Embryos begin to produce lactic acid as soon as they are deprived of oxygen.

This result is clearly established by the data of Figure 2 and Figure 3. There is no indication of a lag in the onset of glycolysis of the sort mentioned by Cohen (1954). But the apparent discrepancy between his results and ours may be resolved by considering the methods used to induce anaerobiosis. In our experiments, the embryos were gassed while spread out in a very thin layer of medium over a relatively large surface; therefore, there was probably little delay in the establish-

ment of anaerobiosis. Cohen's embryos, on the other hand, were gassed in a thicker layer of medium spread over a much smaller surface, and anaerobiosis may have developed more slowly. Indeed, Cohen noted that the period of lag was reduced by increasing the number of embryos per volume of medium: on our interpretation, this is to be expected. Thus, Cohen may be regarded as having shown not a lag in the production of lactic acid after anaerobiosis is attained, but merely a lag in the attainment of anaerobiosis.

(3) For at least four hours of anaerobiosis, embryos younger than 72 hours produce lactic acid at constant rates, whereas the rates of lactic acid production by older embryos under similar conditions are decreasing functions of time (Fig. 2,

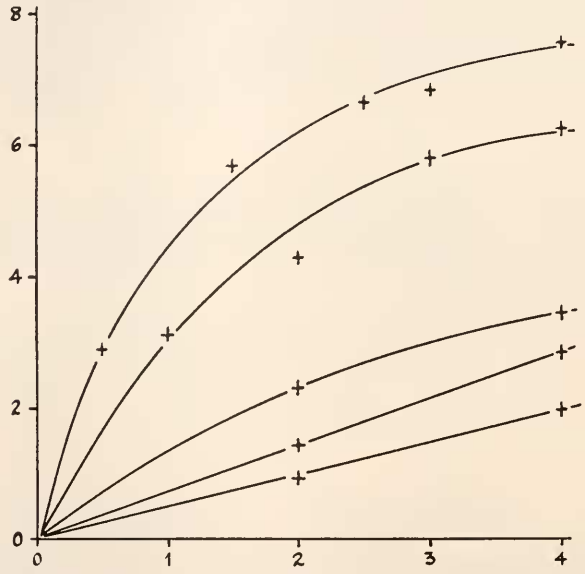

FIGURE 2. Time course of lactic acid production by anaerobic *R. pipiens* embryos, Clutch C. Reading from below to above: Stage 2^{+}, standard age 2 hours; Stages $11\frac{1}{2}$ and 15^{-}, standard ages 38 hours and 66 hours; Stage 18, standard age 96 hours; Stage 19, standard age 118 hours; Stage 20, standard age 140 hours. Abscissa, time in hours. Ordinate, μg. lactic acid per embryo. (Two points near (0, 0) have been omitted. See table in discussion of result (1).)

Fig. 3). But, during the first hour of anaerobiosis, embryos of any age glycolyze at rates which for all practical purposes may be regarded as constant (Fig. 3). The latter result has been obtained in experiments on three clutches of control embryos and two clutches of hybrids, other than that represented by Figure 3.

Among other things, these findings mean that glycolytic rates obtained by estimating the lactic acid contents of post-neurulae at the beginning and at the end of a long period of anaerobiosis (periods of lengths up to 30 hours are not untypical of the sparse literature on the subject) are not strictly comparable with those similarly obtained on pre-neurulae and neurulae. In establishing the data for Figure 4, we have avoided misleading comparisons by taking the rates of glycolysis to be those exhibited during the first hour of anaerobiosis.

(4) The rate of glycolysis of *R. pipiens* embryos is an increasing function of age. The graph relating age to glycolytic rate (Fig. 4) shows that the capacity to glycolyze develops in two phases. The first phase, spanning the interval between fertilization and the onset of neural fold formation, is characterized by a constant rate of glycolysis. The second phase, from the onset of neural fold

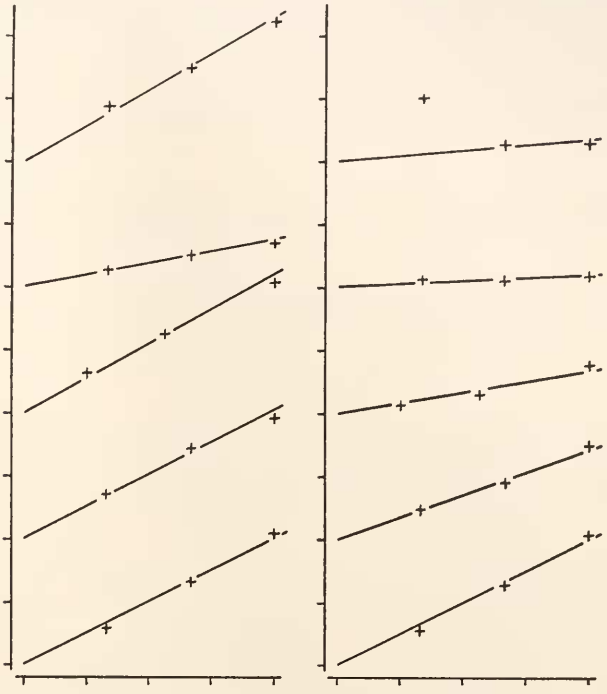

FIGURE 3. Time course of lactic acid production by anaerobic hybrid embryos (right-hand section) and normal control embryos (left-hand section), Clutch F. Reading either section from below to above: Stage 3½, standard age 4 hours; Stage 10½, standard age 30 hours; Stage 13¾, standard age 59 hours; Stage 17½, standard age 90 hours; Stage 19½, standard age 129 hours. Abscissal units lower three curves either section, hours; upper two curves either section, quarter-hours. Ordinal units either section, μg. lactic acid per embryo. (Rates of glycolysis determined from these curves are plotted in Figure 4.)

formation to hatching, is characterized by an exponentially increasing rate of glycolysis. More precisely:

$$g(t)=0.44 \qquad (0\leq t\leq 59)$$
$$g(t)=0.44\, e^{0.0225(t-59)} \qquad (59\leq t\leq 140)$$

where t is the standard age in hours and $g(t)$ is the glycolytic rate in μg. lactic acid per embryo per hour.

It is interesting to note that the acceleratory change in glycolytic rate at 59 hours is paralleled by a similar change in respiratory acceleration at 56 hours (Gregg, 1960.) The close temporal coincidence of the two changes suggests a structural connection of some sort, but details are not yet available.

Comparison with the results of Lennerstrand (1933) and Brachet (1934) is made difficult by complications mentioned in the discussion of results (1) and (3). The data more or less agree with the more circumscribed ones of Barth (1946). But they are clearly inconsistent with those of Cohen (1954), who found that the glycolytic activity of pre-neurulae is a linearly increasing function of age, not a constant function. There is no obvious way to resolve the discrepancy; and, pending further investigation, the two sets of results must remain irreconciled.

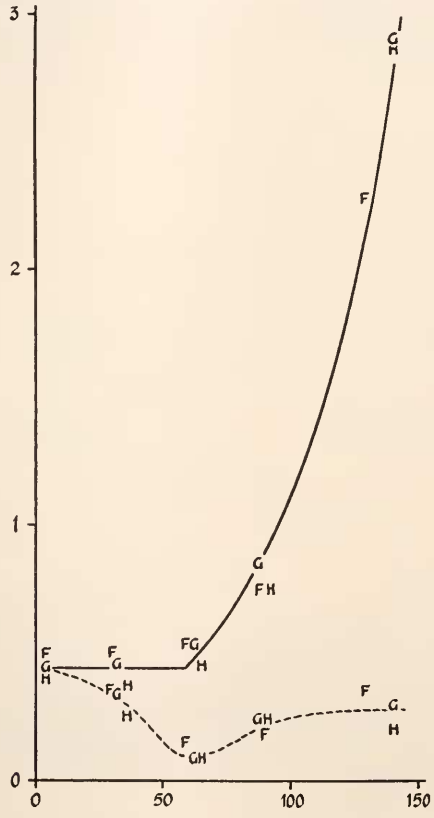

FIGURE 4. Rate of lactic acid production as a function of age, Clutches F, G, H. Lower curve, hybrid embryos. Upper curve, normal control embryos. Abscissa, standard age in hours. Ordinate, μg. lactic acid per embryo per hour. The three points at 4 hours represent both normal and hybrid embryos, whose glycolytic rates at this age are indistinguishable.

(5) Developing hybrid embryos undergo changes of glycolytic rate that are difficult to characterize in any simple way as a mathematical function of age (Fig. 4). Roughly speaking, their capacity to produce lactic acid under anaerobiosis declines from the control value at fertilization to about a fifth of the control value at 59 hours, and then rises slowly until, at 140 hours, the glycolytic rate is about one-eleventh of the control rate. The acceleration at 59 hours may be of the same sort as that in control embryos, but of reduced magnitude. There is no corresponding respiratory acceleration (Barth, 1946). Briefly, our results agree

with those of Barth in showing that hybrid embryos are unable to generate energy by anaerobic glycolysis except at increasingly sub-normal rates. The reasons why are yet unknown.

Summary

1. *R. pipiens* embryos, and gastrula-arrested hybrid embryos obtained by fertilizing *R. pipiens* eggs with *R. sylvatica* sperm, begin to produce lactic acid, without initial lag, as soon as they are deprived of oxygen.

2. Provided that they are younger than about 72 hours (18° C.), embryos of both types are able to sustain the initial rate of glycolysis for at least four hours. Older embryos of both types exhibit a linear production of lactic acid for at least one hour.

3. The development of glycolytic capacity in *R. pipiens* embryos occurs in two phases: one of constant glycolytic rate (0–59 hrs.), the other of exponentially increasing glycolytic rate (59–140 hrs.).

4. The glycolytic rates of hybrid embryos decline from the normal control value at fertilization to about one-fifth of the control value at 59 hours, then increase to about one-eleventh of the control value at 140 hours.

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